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Age Determination of Wrinkled Frog, *Rana rugosa* with Special Reference to High Variation in Postmetamorphic Body Size (Amphibia: Ranidae)

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ABSTRACT—Individual ages of postmetamorphic *R. rugosa* from three populations of Kyoto, Japan (Gondoike, Iwakura, and Kiyotaki), were determined skeletochronologically. From recaptured individuals, one LAG was confirmed to be formed per year. In all three populations, age at sexual maturity was 1–2 yrs in males and 2–3 yrs in females, though some females matured at the age of 1 yr. Longevity was at least 4 yrs in males and 5 yrs in females. Sexual size dimorphism was conspicuous in adults, and females were invariably larger than males. Females had a larger asymptotic size than males but growth rate differed between some localities only in males. In all populations, both sexes showed high variations in SVL and the variation was even higher in juveniles than in adults. This high variation is explained by a prolonged breeding season and resultant mixture of metamorphs with and without overwintering larval stage within a population of this species.

INTRODUCTION

The wrinkled frog *Rana rugosa* is distributed in East Asia from Japan through Korea and northeastern China to southernmost Primorskii, USSR (Maeda and Matsui, 1999; see also in Matsui and Wilkinson, 1992; Hasegawa *et al*., 1999). *Rana rugosa* has been studied from various biological aspects such as phylogenetic position (Matsui and Wilkinson, 1992), enzyme variation (Nishioka *et al*., 1993), sex-determining mechanism (Kashiwagi, 1993; Nishioka *et al*., 1994), antibiotic peptide (Park *et al*., 1994; Suzuki *et al*., 1995), and call variation (Hasegawa *et al*., 1999). Regarding ecology, however, only growth (Okochi, 1978), reproductive behavior (Chang, 1994), and food consumption (Hirai and Matsui, 2000) have been studied, and age estimation, which provides important information about mortality, longevity, and other ecological factors (Paton *et al*., 1991) of this species, has never been made so far.

Skeletochronology is now regarded as an accurate method for determining ages of amphibians (Castanet and Smirina, 1990; Smirina, 1994). The period of hibernation or aestivation yields formation of the lines of arrested growth (LAGs) in the bone of amphibians. In Japanese amphibians, skeletochronology has recently been applied to several species (e.g., *Rana tagoi*: Kusano *et al*., 1995a; *Rana sakuraii*: Kusano *et al*., 1995b; *Hynobius kimurae*: Misawa and Matsui, 1999), and permitted a greater understanding of their life history traits.

Before this technique became popular, individual age had been estimated mainly from body size frequency data (e.g., *Bufo woodhousei fowleri*: Clarke, 1974; *Triturus vulgaris*: Bell, 1977; *Plethodon glutinosus*: Semlitsch, 1980). If the intrapopulation variation in life history is very small, this may be usable. However, for a species that exhibits high variation in intrapopulation life history, this method cannot apply first of all. *Rana rugosa* belongs to this category and skeletochronology is expected to play a great power in this species. *Rana rugosa* is a prolonged breeder with its breeding season from May to September and, further, it usually lays eggs more than once in one breeding season (Maeda and Matsui, 1999). This variation in the timing of spawning results in the occurrence of metamorphs with and without larval overwintering within a population. In order to clarify age and SVL variations among and within populations of *R. rugosa*, we applied skeletochronological technique to three populations from Kyoto, Japan. These three populations are from different environmental conditions; reservoir, paddy field, and river shoreline.

MATERIALS AND METHODS

Sample collection

The frogs were collected between 3 May 1995 and 12 September 1999 from three localities with different environmental conditions, especially of the water, in Kyoto Prefecture, Japan: reservoir at Gondoike, Iwakura (hereafter referred to Gondoike; with a large body of permanent still-water); paddy field at Muramatsu, Iwakura (Iwakura:

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the water was artificially drawn and not permanent, but tadpoles could flow into a small stream nearby and hibernate there), and river shoreline at Kiyotaki (Kiyotaki; moderate sized river with flowing water and never dried up). Besides the water level, these three localities differed in prey types of metamorphosed frogs, and part of the samples used in this study are those obtained for food habit studies (Hirai and Matsui, 2000). *Rana rugosa* bred in all these localities from middle of May to the end of August. Tadpoles hatched earlier metamorphosed within a year mostly by September and sometimes by early October, but those hatched later overwintered and metamorphosed in the next year by July.

In all the three habitats, frogs were captured by hand, weighed, measured for snout to vent length (SVL), marked by toe clipping, and released at the place of capture. When already marked frogs were recaptured, we cut intact digits to examine the formation of additional LAGs over a time. Clipped digits were preserved in 10% formalin for later examination.

The presence or absence of secondary sexual characteristics such as nuptial pads and vocal sacs, and calling behavior were used to determine maturity of males. It was much more difficult to determine maturity in females, and only frogs found in amplexus or those released eggs when handled could be confirmed as mature. We treated individuals that had larger SVL than mean SVL of adult males but showed no male secondary sexual characters as subadult females.

Skeletochronology

A total of 488 preserved digits including 216 (78 juveniles, 75 adult males, 17 subadult females, and 46 adult females) from Gondoike, 140 (55 juveniles, 64 adult males, 7 subadult females, and 14 adult females) from Iwakura, and 132 (13 juveniles, 81 adult males, 2 subadult females, and 36 adult females) from Kiyotaki were analyzed. In addition to these, six overwintered tadpoles were collected on 12 May 1999 at Kiyotaki (n=1) and on 25 April 2000 at Gondoike (n=5) to examine occurrence of LAGs in their femurs and toe bones. In order to examine variation in the number of LAGs within an individual, bones from fingers and toes were examined in three frogs from Iwakura. Bones were washed in running water for 24 hr, decalcified in 5% nitric acid for 60–90 min, and washed again in the running water for 24 hr. Each digit was cross-sectioned (20–22 mm thick) by a freezing microtome and stained with hematoxylin (Mayer's acid hemalum) for 30 min. Since resting line was absent or incomplete in the epiphysis, sections from the central region of diaphysis were selected, and mounted in glycerin after rinsed in the tap water. We examined sections under a light microscope, took the picture for further analysis, and counted the number of lines of arrested growth (LAGs) present in the periosteal bone. To specify the age of each individual frog, the time of capture in the year was taken into consideration. The frogs caught in May in the beginning of the breeding season had not started formation of a new line, and one year was added in estimating their ages.

Analysis

Because *R. rugosa* breeds and metamorphoses over several months, it is difficult to set the starting point in calculating ages. We thus arbitrarily set the starting point on 1 May at midpoint of metamorphosis.

We examined sexual differences in age and age composition of adult frogs by Dunn's nonparametric multiple comparison test (Zar, 1996). For differences in diameters of resorption line (RL) and LAG, and in intersexual body size, Tukey multiple comparison test (Zar, 1996) was applied. The significance level was set at 0.05. Logistic model $L=a/(1+e^{c-bt})$, where L is the SVL (mm) at time t (yr); a is the asymptotic size; c is the constant determined by putting 0 to t; and b is the instantaneous growth rate, was used to estimate the growth pattern (Zar, 1996). In this estimation, we used data of unsexed juveniles for both sexes. Following Zar (1996), we used estimates of the parameters provided by a software KaleidaGraph 3.0.4. Sexual Dimorphism Index (SDI: Miaud *et al.*, 1999) was determined as the mean SVL of females/mean SVL of males.

RESULTS

General result of skeletochronology

In the cross-sections of digit bones there were dense lines each separated by a clear zone and these were interpreted as LAGs (Fig. 1). A resorption line was also found but it was clearly distinguished from LAGs. Actually, the diameter of RL was always smaller than that of the first LAG, and both showed no variation among different age groups (Tukey test, $P>0.05$). We could not observe metamorphosed line in any sections.

In all the three individuals from Iwakura, the number of LAGs did not differ among different digits of fingers and toes and indicated that any digit could be used for studying age of *R. rugosa* by skeletochronology. The LAGs were not different in the distinctness and intensity in all the populations.

We could not find any lines in femurs or toe digits of al the six overwintered tadpoles examined. All juvenile frogs collected in autumn (n=18, end of August to October) and more than half (n=49 of 90 individuals) caught in May after winter hibernation had no LAGs. On the other hand, the remaining 41 individuals in May and all juveniles collected later in June (n=35) had a LAG in their bones. These results indicated that LAG was formed several months after emerging from hibernation.

Eighty out of 830 marked individuals were recaptured over a year, of which we examined LAGs for four frogs that were collected twice with one overwintering period in between. All these four had one additional LAG in the year following hibernation (Table 1). These results indicated that the number of LAG represented actual age of postmetamorphic frogs (i.e., excluding larval age).

Postmetamorphic age of individuals

What is very important is that the age we could estimate from LAG represents the period the frog experienced after metamorphosis (i.e., postmetamorphic age), while we could not know actual individual age, or whether or not the frog overwintered at the larval stage.

Because adult frogs were collected over a prolonged breeding season from May to August, ages after the metamorphosis arbitrarily set at the beginning of May (see above) differed according to the time of collection. We therefore further adjusted the age of each individual to the middle of the breeding season (15 July). The median of ages thus obtained did not differ between sexes in Iwakura population (2.21 yrs in both sexes) but females were significantly older than males in Gondoike (1.21 yrs in males v.s. 2.21 yrs in females) and Kiyotaki (2.21 yrs in both sexes) populations (Dunn's test, $P<0.05$). In the latter population, medians were same between the sexes, but the pattern of age composition differed significantly (see table 2). In neither sex, the age composition differed between the three populations (Dunn's test, $P>0.05$).

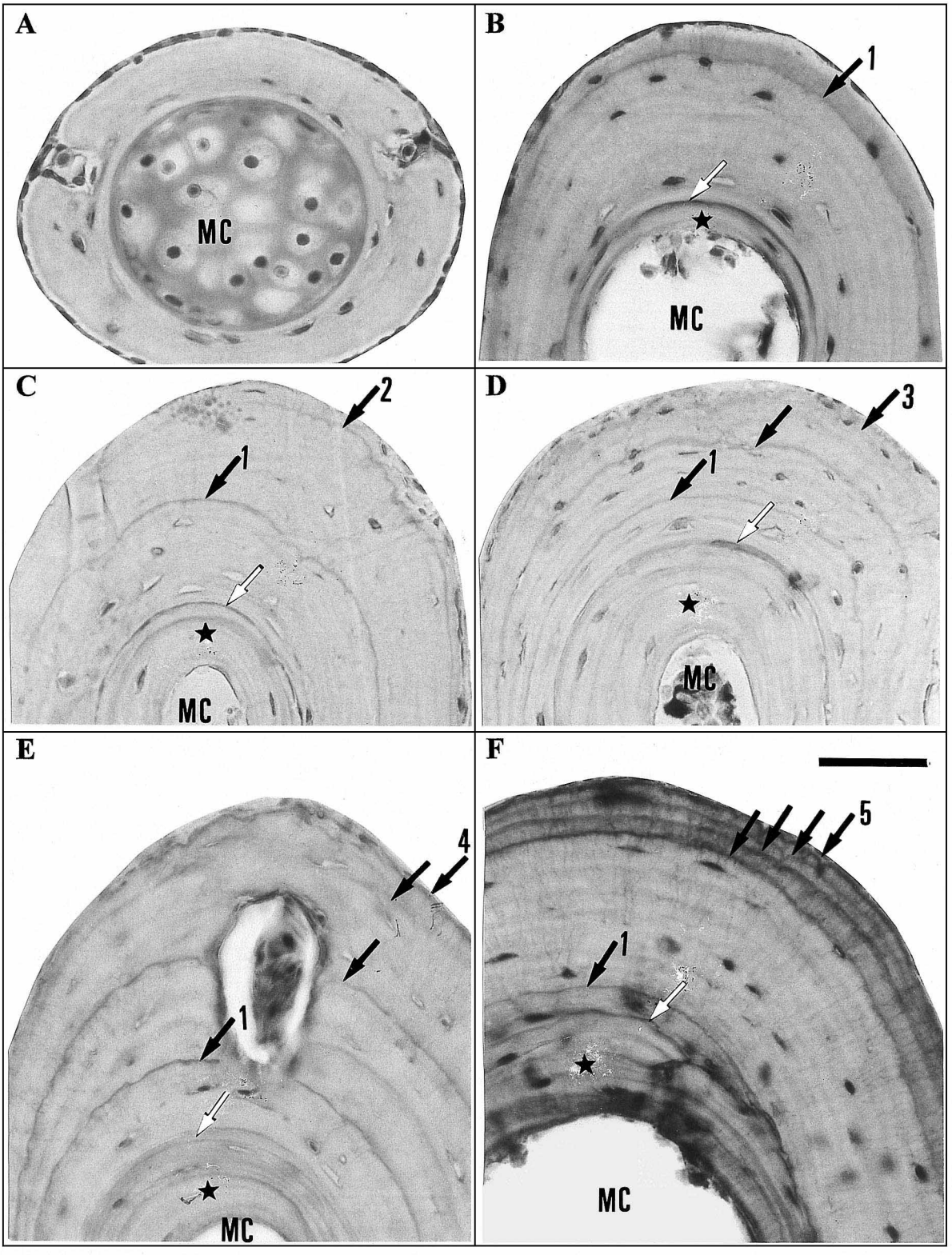

Fig. 1. Phalangeal cross-section of *Rana rugosa*; (A) juvenile with 21.5 mm SVL collected on 29 August 1997 showing no LAG, (B) juvenile with 27.5 mm SVL collected on 12 May 1997 showing 1 LAG, (C) male with 40.0 mm SVL collected on 29 June 1997 showing 2 LAGs, (D) male with 45.0 mm SVL collected on 9 June 1998 showing 3 LAGs, (E) female with 56.1 mm SVL collected on 9 June 1998 showing 4 LAGs, (F) female with 56.2 mm SVL collected on 23 June 1997 showing 5 LAGs. MC=marrow cavity, black arrows=LAGs, white arrow=resorption line, black star=endosteal bone, scale bar=50 μm.

Table 1. Body size growth and LAG increase in recaptured frogs. M=males, F=females.

Individual No.	At 1st capture			At recapture		
	Date	SVL (mm)	N of LAG	Date	SVL (mm)	N of LAG
M4090	8 June 1998	36.5	1	29 May 1999	43.8	2
M4171	19 August 1998	40.4	1	5 June 1999	42.5	2
F4120	27 June 1998	58.9	2	5 June 1999	62.0	3
F4122	3 July 1998	58.0	2	6 July 1999	61.1	3

Age at maturity and longevity

As shown in Table 2, the youngest adult frogs belonged to the age class of 1 yr in both sexes in all populations. However, in Gondoike and Iwakura, immature individuals outnumbered adult males in the 1 yr age class (Fig. 2, Table 2). Similarly, 1 yr old adult females occupied much smaller proportions than 2 yrs old individuals in all populations, and not a small number of subadult females were estimated to be 2 yrs old in Gondoike and Iwakura (Table 2). These situations somewhat differed in Kiyotaki; more adult males were found than immature individuals in the 1 yr age class, and subadult females were much less in number than adults in the age class of 2 yrs (Table 2). Overall, sexual maturity was usually attained from 1 to 2 yrs of age in males and 2 to 3 yrs in females in this species. On the other hand, males of 4 yrs were the oldest in Iwakura and Kiyotaki populations, while the oldest female, 5 yrs old, was found in Iwakura (Table 2).

Table 2. SVL (in mm) variation in each age class. J=juveniles, M=males, SF=subadult females, and F=females.

Population	Age class	SVL				
		N	Range	Mean	2SE	CV
Gondoike	J0	9	21.5–30.0	24.40	1.97	11.7
	J1	69	23.9–40.5	30.28	0.77	10.6
	M1	41	35.5–45.0	40.13	0.67	5.4
	M2	29	37.5–45.6	41.83	0.67	4.3
	M3	5	40.0–49.0	46.10	3.17	7.7
	SF1	6	42.3–49.8	45.78	2.07	5.6
	SF2	11	40.0–50.9	46.85	1.59	5.6
	F1	3	51.5–52.3	51.83	0.50	0.8
	F2	31	49.1–58.7	53.02	0.87	4.6
	F3	10	51.1–60.0	56.01	1.83	5.2
	F4	2	51.0–56.1	53.55	–	–
Iwakura	J0	7	20.9–30.8	26.64	2.59	13.9
	J1	48	23.1–35.5	29.23	0.99	11.8
	M1	29	29.6–43.5	38.30	1.20	8.5
	M2	22	40.8–46.7	43.29	0.68	3.7
	M3	11	43.9–47.4	45.15	0.73	2.7
	M4	2	44.9–45.0	44.95	–	–
	SF1	3	38.0–44.0	40.50	3.61	7.7
	SF2	4	44.8–49.0	47.77	1.24	3.2
	F1	2	44.9–53.8	49.35	–	–
	F2	11	49.0–59.0	53.01	1.98	5.6
	F3	–	–	–	–	–
	F4	–	–	–	–	–
	F5	1	–	56.20	–	–
Kiyotaki	J0	2	20.0–25.1	22.55	–	–
	J1	11	30.0–36.1	33.31	1.34	6.7
	M1	31	36.4–44.8	40.93	0.81	5.5
	M2	30	38.4–46.0	43.34	0.70	4.4
	M3	18	43.9–48.0	45.96	0.70	3.2
	M4	2	46.2–49.5	47.85	–	–
	SF1	1	–	50.10	–	–
	SF2	1	–	48.80	–	–
	F1	1	–	59.10	–	–
	F2	19	52.0–64.8	56.83	1.58	6.0
	F3	12	53.9–62.0	58.04	1.65	4.9
	F4	4	53.8–61.5	58.13	3.97	6.8

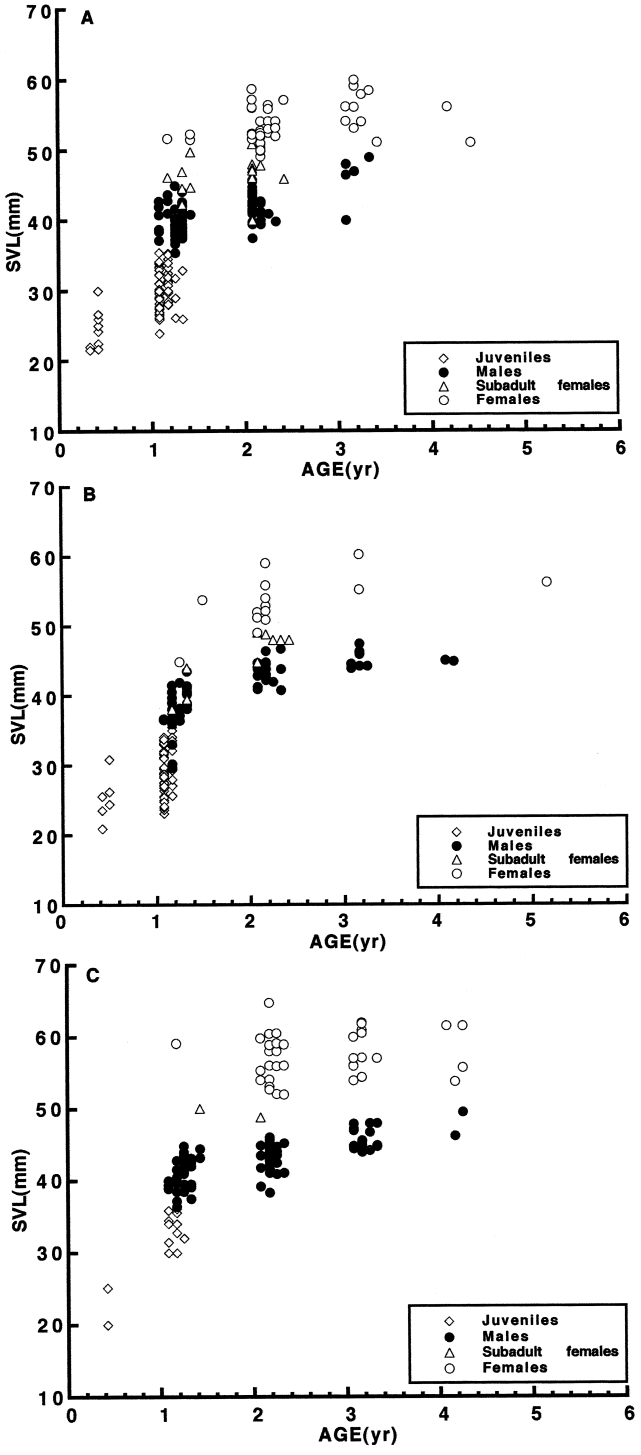

Fig. 2. SVL distribution of *Rana rugosa* in each age group for (A) Gondoike, (B) Iwakura, and (C) Kiyotaki populations.

Body size and variation

As shown in Table 3, adult *R. rugosa* showed clear sexual size dimorphism (SSD) in which SVL of females was significantly larger than males in all populations (SDI = 1.29-1.33; Tukey test, $P<0.05$). In both Gondoike and Iwakura, both sexes were significantly smaller than in Kiyotaki (Tukey test, $P<0.05$), while Gondoike and Iwakura were not significantly different in SVL (Tukey test, $P>0.05$).

Variation in SVL distribution was high in both sexes of adults in all populations (Fig. 2). The coefficient of variation (CV) in SVL was even higher in juveniles (CV=6.7–13.9) than

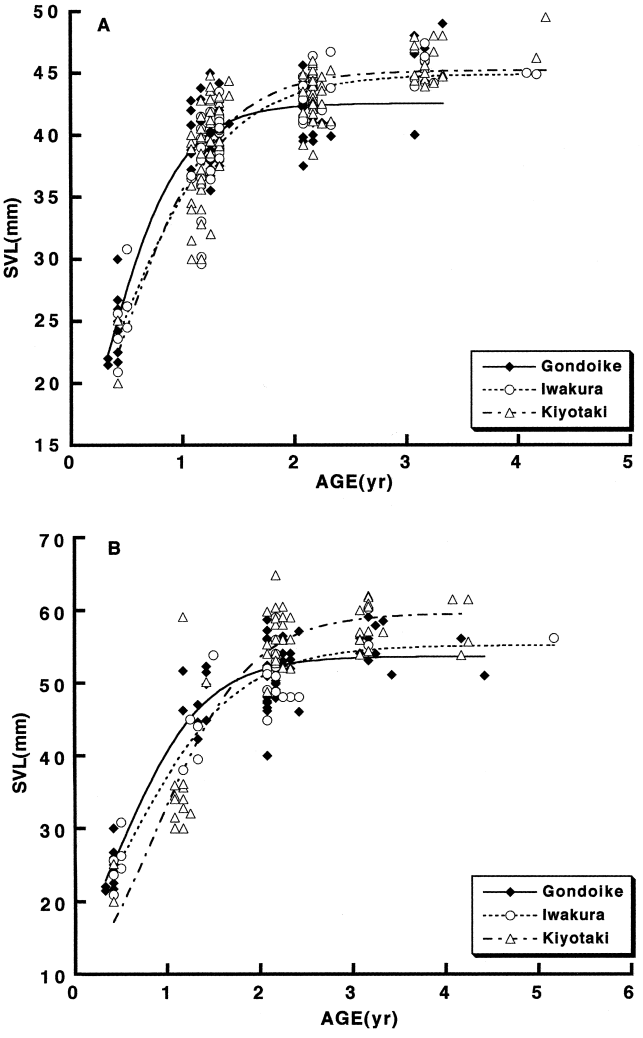

Fig. 3. Growth pattern of *Rana rugosa* from Gondoike, Iwakura, and Kiyotaki populations. (A) males, (B) females.

Table 3. Sexual dimorphism in SVL (mm) of adult frogs. SDI = Mean SVL of females/Mean SVL of males.

Population	Males			Females			SDI
	N	Mean±2SE	Range	N	Mean±2SE	Range	
Gondoike	75	41.2±0.60	35.5–49.0	46	53.2±0.82	49.1–60.0	1.291
Iwakura	64	41.4±0.94	29.6–47.4	14	53.4±2.20	44.9–60.2	1.289
Kiyotaki	81	43.1±0.60	36.4–49.5	36	57.4±1.08	52.0–64.8	1.332

Table 4. Asymptotic size in mm and instantaneous growth rate (mean±2SE) of frogs from three populations, estimated by logistic model.

Population	Asymptotic size		Instantaneous growth rate	
	Males	Females	Males	Females
Gondoike	42.5±0.93	53.7±1.61	2.98±0.631	2.14±0.523
Iwakura	44.9±1.05	55.2±3.82	1.99±0.333	1.70±0.561
Kiyotaki	45.2±1.00	59.6±2.84	2.28±0.531	1.95±0.620

in adults (CV=0.8–8.5) as shown in Table 2. These results surely derived from the fact that frogs which metamorphosed before and after hibernation are mixed in juveniles of each population.

Growth pattern

To analyse the growth pattern in *R. rugosa*, growth parameters were estimated by the logistic model. As shown in Fig. 3, growth rate of males suddenly decreased when they reached 1 yr of age, while, that of females decreased at the end of 1 yr or after they reached 2 yrs old (Fig. 3). In all populations, females had a larger asymptotic size than males, and in the same sex, asymptotic size was not different among the populations in females but in males both of Iwakura and Kiyotaki were larger than Gondoike (Table 4). The instantaneous growth rate did not differ between the sexes within a population. In males, Gondoike had a higher rate than Iwakura (Tukey test, $P<0.05$), but females did not differ between populations in this rate (Table 4).

DISCUSSION

Skeletochronology has been proved to be reliable in studying age of amphibians (Paton *et al.*, 1991; Caetano and Castanet, 1993; Castanet *et al.*, 1996). In all amphibian species from the temperate region hitherto studied by skeletochronology, one LAG is formed after they experienced low temperatures in the winter inactive season (e.g., Paton *et al.*, 1991; Misawa and Matsui, 1999). Our result of skeletochronology for postmetamorphic *R. rugosa* coincided with these previous results, and formation of one LAG per one year was confirmed.

Unfortunately, we could estimate neither actual age including larval stages, nor presence or absence of overwintering experience at the larval stage for each individual frog. Platz *et al.* (1997), in reporting LAG of *Rana subaquavocalis*, mentioned to the overwintering tadpoles. They, however, directly counted the number of LAGs found in metamorphs as their age without discussing LAGs of these tadpoles. As far as we know, no studies have ever been made to examine LAGs in overwintered anuran tadpoles. In *R. rugosa*, we could not detect any LAGs in either femur or phalangeal bones of overwintered tadpoles from two populations. We recently also confirmed that overwintered tadpoles of *R. catesbeiana* had no lines in the bone sections (Khonsue and Matsui, 2001). From these lines of information, we surmise that LAGs are not formed in overwintered larvae at least in ranid frogs found in Japan. This strongly contrasts with urodelan larva in which clear LAGs are formed after hibernation (Misawa and Matsui, 1999), and suggests the presence of physiological differences between these two orders.

Plytycz and Bigaj (1993) argued that femur bones were more suitable for accurate age determination than digits in *Bombina variegata*. Misawa and Matsui (1999), however, reported that LAG numbers did not differ among digital, femoral, and humeral bones in *Hynobius kimurae*. In this study, we demonstrated that different digits are similarly suitable for skeletochronological study. In this way, we could use digits collected by mark-recapture studies successfully for age determination. This avoidance of killing animals and acquisition of information necessary for life history studies meet to the requirement of conservation biology.

At present, mark-release-recapture method and skeletochronology are regarded as reliable for age determination of amphibians (Halliday and Verrell, 1988; Castanet and Smirina, 1990), although there are some contradictions (Plytycz and Bigaj, 1993). In this study, we coupled skeletochronology with mark-recapture method to estimate age of *R. rugosa* and confirmed that the results of the two methods are similar.

Age at maturity and longevity differ depending on species and localities. In *R. rugosa*, 1–2 yrs in males and 2–3 yrs in females were needed to sexually mature. These estimations well conformed to the pattern of growth curves in which growth ratio quickly dereased after these years. Many males were still immature at the age of 1 yr in Gondoike and Iwakura, but males mostly matured at the age of 1 yr in Kiyotaki. Similarly, some females actually began reproduction as early as at the age of 1 yr in all populations. In this way, *R. rugosa* can be regarded as highly variable in the timing of sexual maturity. The interpopulation variation in the age at maturity may be resulted from differences in the quality of habitat or environment as proposed by Augert and Joly (1993). In addition, some genetic factors may be also related to these differences. On the other hand, the longevity in *R. rugosa* is short, only 3–5 yrs in nature. Although the maximum longevity did not differ between sexes in Kiyotaki, this might be a result of small sample size, and females of this species are regarded to have longer longevity than males.

Adults of *R. rugosa* exhibited SSD in all three populations, and the SDI values we obtained (1.29–1.33) were only slightly larger than those reported by Fukuyama and Kusano (1989) for populations from the Kanto region (1.25–1.28). Compared with many other Japanese species, this degree of

SSD in *R. rugosa* seems to be not small (Fukuyama and Kusano, 1989). Classically, prolonged breeders were surmised to be selected to increase male body size (hence smaller SDI; e.g., Woolbright, 1983), but nowadays, selection on male body size is considered to be not so simple; it may be constrained by a diversity of ecological and demographic factors (Halliday and Tejedo, 1995).

Fukuyama and Kusano (1989), in reporting SSD of *Buergeria buergeri* which shows even greater SDI than in *R. rugosa*, considered the selection to favor medium-sized but fatty males rather than larger but slender ones, by which process marked SSD in *B. buergeri* might induced. This explanation may hold for *R. rugosa*, although we have currently no data to analyze fatness. Additionally, slightly larger SDI in Kiyotaki than in other two populations might be related to its torrential breeding habits; male smaller size might be advantageous to mated pairs to reduce resistance, and to females to have a lighter load while swimming about, in the flowing water (Matsui and Matsui, 1990).

Both sexes of adults were on an average larger in Kiyotaki than in Gondoike and Iwakura, although intrapopulation variation in body size was high in all populations. This variation may be also related to unique, lotic habitat of Kiyotaki population. We found that the juveniles were much more variable in SVL than adult frogs. Similar phenomenon has been observed in populations other than Kyoto, and Okochi (1978) reported that metamorphs collected in July was significantly larger (mean size of 25.8 mm) than in August (20.8 mm) in a population from Chiba. This must be resulted from the fact that *R. rugosa* is a prolonged breeder (Chang, 1994) and some tadpoles overwinter and others do not (Maeda and Matsui, 1999). This means that the metamorphs within a population are mixture of frogs that experienced different life histories. Frogs treated here as juveniles actually contained those with and without one LAG and indicated heterogeneous age composition within a population. Skeletochronological method is especially powerful for species like *R. rugosa* whose body sizes are too variable to analyze growth and population composition from size-frequency data.

In all populations of *R. rugosa*, females had growth rates similar to males, but reached to a lager asymptotic size than males through continuous growth in a longer period. As shown above, there was a difference between the sexes in the age of maturation, and this difference resulted in females with delayed sexual maturity, growing faster thereafter, and reaching a larger asymptotic size than in males. This is one of the four types of ways in which SSD may arise (Halliday and Tejedo, 1995). A larger growth rate in males in Gondoike than in Iwakura may have been caused by different water level (permanent and temporary, respectively) and/or different food available (Hirai and Matsui, 2000), and lack of difference in females between these two populations may be a result of small sample size.

Being a prolonged breeder and lays multiple clutches, *R. rugosa* seems to be reducing the opportunity of unsuccessful clutch in a year and the hence risk of population disappearance. This species also has an unpalatable skin secretion that keeps them from predators (Mori, 1989), inhibits growth and activity of bacteria (Suzuki *et al*., 1995), and controls pathogenic microorganisms (Park *et al*., 1994). However, as documented in the present study, the species actually has a short longevity with a smaller number of older individuals within a population. Thus, although populations of *R. rugosa* seems to be currently not much unstable, the species is not free from the risk of population disappearance if the environmental conditions are not suitable for reproduction in continuous years. In fact, in paddy fields at Iwakura, the activity of frogs, especially reproduction, highly depends on farmers' control of water. We consider that long-term studies of LAGs in the bone of *R. rugosa* would prove powerful in charting the population rise and decline in relation to impact of human activities as proposed by Kellner and Green (1995).

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